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Antimicrobial activity of aqueous extracts of sea cucumber (*Isostichopus badionotus*) from the coast of Yucatan, Mexico

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The antibacterial activity of aqueous extracts from different tissues of *Isostichopus badionotus* was evaluated in this study. The antibacterial activity was detected in aqueous extracts of muscle and respiratory tract. A high antibacterial activity was found in the Membrane Protein fraction (MP) muscle on *Staphylococcus aureus*, *Vibrio cholerae*, *Escherichia coli* and *Pseudomonas aeruginosa*. The minimum inhibitory concentration (MIC) of MP against *S. aureus* was 37.4 and 8.4 µg/ml protein for *V. cholerae*. It also showed resistance to treatment with proteinase K and heat, indicating that the active compound of this fraction is from non-protein origin. The results of this study suggest that *I. badionotus* is a potential source for discovering new antibiotics.

Key words: *Isostichopus badionotus*, aqueous extracts, antimicrobial activity, sea cucumber, *S. aureus*, *V. cholerae*.

INTRODUCTION

Nowadays, the continued use of antibiotics in disease control has resulted in multidrug-resistant bacterial strains worldwide and, as expected, hospitals have become a breeding ground for pathogenic microorganisms associated to human diseases (Al-Haj et al., 2010; Haug et al., 2002b). In particular, *Staphylococcus aureus* causes illness in intensive care units of hospitals around the world. This bacterium acquires new virulence factors by developing resistance to new antibiotics. Thus, *S. aureus* resistant to methicillin (MRSA) has spread in intensive care units at hospitals. MRSA is present in 40 to 70% of all diseases that were found in intensive care units (Mariana et al., 2009; Zetola et al., 2005), and infection is higher in skin, soft tissue, respiratory system causing vascular disorders and it is also associated to joint disorders (Al-Haj et al., 2009). *Vibrio cholerae* is a gram-negative bacteria and its pathogenic effect is due to the production of multiple toxins (Prats, 1987; Panigraphi et al., 1990). It has a worldwide distribution and environmental studies have shown that marine environments typically found in bays and estuaries (Morris and Black, 1985). The main source of contamination is by ingestion of raw foods from marine origin, clinical manifestations result gastrointestinal and extraintestinal infections such as biliary tract (Prats, 1987; Forné et al., 1987). It therefore requires new natural sources of antimicrobial

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agents to combat them. In this sense, marine organisms can be an alternative for the discovery of antimicrobial compounds to develop new drugs (Haug et al., 2002a; Mariana et al., 2009). The echinoderms are benthic organisms, which are constantly exposed to a high number of fungi, bacteria and viruses, which can be harmful to them. The survival of these organisms depends on efficient mechanisms for the production of antimicrobial substances to protect themselves to microbial infections (Haug et al., 2002b). Sea cucumbers belonging to the phylum Equinodermata, also known as "Gamat" in Indonesia are used in traditional remedies to cure asthma, hypertension, rheumatism, burns, internal and external wounds, and have been used to combat multiresistant bacteria (Mariana et al., 2009; AL-Haj et al., 2010).

Haug et al. (2002b) examined and found *Cucumaria frondosa* high antibacterial activity in the fractions of eggs, and muscle coelomocytos against *Corynebacterium glutamicum* and gram-positive bacteria. Another study by Mariana et al. (2009) found that methanol extract of *Stichopus badionotus* inhibited the growth of MRSA. In subsequent studies, AL-Haj et al. (2010) found in methanolic extracts of *G. changií* inhibited growth of *S. aureus* and *S. pyogenes.* On the coast of Yucatan, not much is known about these organisms, but different species have been described for sea cucumber. The most abundant and largest is *Isostichopus badionotus*. International demand has meant that this organization is about to explode, creating an imbalance in natural populations. Therefore, it is important to conduct studies to identify the potential of this organism in the search for new drug products. In this paper, a comparison of antimicrobial activity in different organs or tissues of *I. badionotus* and preliminary characterization of antibacterial components was performed.

MATERIALS AND METHODS

Collection of animals

The samples of *I. badionotus* were collected on the coast of Yucatan, Mexico in April to June 2010. Sea cucumbers were transported in ice coolers to the laboratory of Aquatic nutrition to dissect the organisms and to separate the organs and tissues. The organisms were divided into gonads, muscle and respiratory tract. All samples were frozen at -20°C for subsequent lyophilization.

Extracting and separation of antibacterial factors

Lyophilized samples (3 to 30 g) were extracted with 10 vol (v/w) of phosphate buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO4, 10 mM KH_2PO_4, 27 mM KCl) for 12 h at 4°C. The extract was centrifuged at 27, 000 *g* for 15 min at 4°C. For each sample, the supernatant was collected and stored at 4°C; the residue was extracted again by stirring for 4 h at 4°C. The combined supernatant was designated crude extract (CE) and centrifuged at 100, 000 *g* for 45 min at 4°C. The supernatant was recovered and termed termed soluble fraction (SP). The precipitate was dissolved in PBS, supplemented with 0.1% Tween 20 and designated as membrane fraction (MP) (Meyer et al., 1996). The different fractions obtained were stored at -20°C. Total protein was determined by the method of Bradford (1976). Then its antimicrobial activity was evaluated against different microorganisms.

Conditions for growth of microorganisms

Gram-negative bacteria *Vibrio cholerae* (CAIM 591), *V. mimicus* (CAIM 602), *V. alginolyticus* (CAIM 516), *V. vulnificus* (CAIM 610), *V. parahaemolyticus* (CAIM 320) were obtained from the Collection of Aquatic Microorganisms Important, Center for Nutrition and Development, A. C., Mazatlan Unit for Aquaculture and Environmental Management, *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) and Gram-positive bacteria *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212) and the fungus *Candida albicans* (ATCC 10231) were used for antimicrobial evaluation. The organisms were grown in Trypticase soy Agar (TSA, DIBICO, SA de CV Mexico City, Mexico) at 37°C for 18 to 24 h. In the case of *Vibrio* species were added with 0.5% (w / v) NaCl in the middle TSA.

Evaluation of antimicrobial activity

The methods used were those described by Duvick et al. (1992) and Meyer et al. (1996). A suspension of bacteria according to the McFarland scale to 0.5 (~ 1×10^8 CFU). We took 200 µl of this suspension and inoculated petri dishes containing 20 ml of medium TSA; were then placed sterile paper disks (3MM, Whatman) of 8 mm in diameter and were applied 1 and 2.6 mg/ml of protein CE, SP and MP of the different tissues of *I. badionotus*. As a positive control we used ampicillin (0.4 mg/ml) and PBS buffer as a negative control. Finally, Petri dishes were incubated at 37°C and the halo of inhibition was observed after 24 h. For *C. albicans* was adjusted optical density (Abs_{600} = 0.1365 nm, equivalent to 1×10^6-5×10^6 CFU) and used the same conditions described earlier. All experiments were performed in duplicate.

Determination of minimum inhibitory concentration (MIC)

The MIC was determined by the MP of muscle in the presence of *S. aureus* and *V. cholerae*. Tubes with 500 µl of Trypticase soy broth 2x (TSB, Bioxon, Becton Dickinson of Mexico) and 440 µl of H_2O. Then placed 50 ul of MP at a concentration of 50, 25, 12.5 and 6.25 µg/ml protein. The mixture was supplemented with 10 ml of bacterial suspension to 1×10^8 CFU. The contents of each tube was mixed and incubated in an incubator at 37°C for 24 h. Bacterial growth was determined by optical density at 600 nm using an Eppendorf BioPhotometer. The MIC was defined as the lowest concentration of MP which decreases the optical density of bacterial growth, this compared with the optical density of growth control (bacteria grown in TSB medium only). The experiments were performed in duplicate. Ampicillin was used as positive control at a concentration of 0.4 mg/ml.

Treatment with proteinase K and heat

The MP muscle was evaluated by sensitivity to proteinase K. Proteinase K (Promega) was dissolved in H_2O at a concentration of 10 mg/ml. It took 12.5 µl of proteinase K solution and added 12.8 µl of MP containing a concentration of 4.7 mg/ml protein. The mixture was supplemented with 37.2 µl of H_2O and incubated at 42°C for 90

Table 1. Antimicrobial activity in extracts from sea cucumber (*I. badionotus*) against *Candida albicans* (*C. a.*), *Escherichia coli* (*E. c.*), *Enterococcus faecalis* (*E. f.*), *Staphylococcus aureus* (*S. a.*), *Staphylococcus epidermidis* (*S. e.*) and *Pseudomonas aeruginosa* (*P.* a.).

Órgan/tissue	Fraction	Antimicrobial activity					
		C. a.	***E. c.***	***E. f.***	***S. a.***	***S. e.***	***P. a.***
Body	CE	-	++	-	++	++	++
	SP	-	++	-	++	++	++
	MP	-	++	-	++	++	++
Respiratory organs	CE	-	+	-	+	+	-
	SP	-	+	-	+	+	-
	MP	-	++	-	++	+	-
Gonads	CE	-	-	nt	-	nt	nt
	SP	-	-	nt	-	nt	nt
	MP	-	-	nt	-	nt	nt

-, No antimicrobial activity at a protein concentration of 2.6 mg/ml; + antimicrobial activity at a protein concentration of 2.6 mg/ml; ++ antimicrobial activity at a protein concentration of 1 mg/ml; nt, not tested, crude extract (CE), soluble protein (SP), membranal extracts (MP).

min. The temperature was raised to 85°C for 15 min to inactivate proteinase K. As a control (heat treatment) 12.5 µl of H_2O was added to 12.8 µl of diluted MP was subjected to the same treatment of the sample treated with proteinase K (Haug et al., 2002a, b). Ampicillin (0.4 mg/ml) was used as a positive control to compare the activity of proteinase K. The antimicrobial activity of the treated samples was determined by the agar disk diffusion method of paper, as described earlier. The experiments were performed in duplicate.

RESULTS

The results show the *in vitro* antimicrobial activity of species *I. badionotus*, which is found in muscle and respiratory tract with most of the bacteria tested. However, when the antimicrobial activity was compared in the different fractions, differences were found in fractions and organs tested (Tables 1 and 2). Of the most sensitive microorganisms evaluated were *E. coli*, *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *V. cholerae*, while the *C. albicans* and *E. faecalis* were the least sensitive. High activity in muscle was found in all fractions to a protein concentration of 1 mg/ml, on the bacteria *E. coli*, *S. aureus*, *S. epidermidis*, *P. aeruginosa* and most species of *Vibrio*. The antimicrobial activity was not found with the strains *C. albicans* and *E. faecalis* (Tables 1 and 2). With the respiratory tract we found high activity with the MP in which inhibited the growth of *E. coli*, *S. aureus* and *Vibrio* sp. species. In contrast, SP and CE fractions showed low antimicrobial activity with *E. coli*, *S. aureus* and *S. epidermidis* (Tables 1 and 2). The activity against *C. albicans*, *E. faecalis*, *P. aeruginosa* and *Vibrio* sp. species was not detected in the fractions of this tissue. In contrast, the gonads tissue presented no antimicrobial activity in the fractions evaluated. In general, high activity was found in muscle tissue, especially in the MP, where activity was observed with most of the bacteria (Tables 1 and 2). It was therefore decided to find the minimum inhibitory concentration (MIC) of this fraction in the presence of the bacteria *V. cholerae* and *S. aureus*.

The MIC of the MP of *I. badionotus* against *S. aureus* was 37.4 µg/ml, was higher than the bacteria *V. cholerae* was 8.4 µg/ml protein (Table 3). Figure 1 shows the antibacterial effect of the MP during the time of growth of *V. cholerae*. Low antibacterial effect was evident at a concentration of 8.4 µg/ml protein and very low decrease in growth was found at a concentration of 33.6 µg/ml protein. However, in control of ampicillin showed no changes in growth (Figure 1). The results of treatment with proteinase K and heating at the MP of muscle are shown in Figure 2, which was not sensitive to such treatments tested against *V. cholerae* (Figure 2) and *S. aureus*.

DISCUSSION

A selection of antibacterial activity in different organs or tissues of *I. badionotus* was performed. The results show that the species has evaluated antibacterial activity *in vitro*. This present study demonstrated the presence of antibacterial factors in the muscle tissues and the respiratory tract, but not in the gonad tissue. In similar work in sea cucumbers, they have found compounds with antimicrobial activity in muscle methanol extract of *G. changii*, *S. baionotus*, *B. mormorata* and *P. parvimensis*, which showed an inhibitory activity on the growth of *S. aureus*, *S. pyogenes*, *V. harveyi*, *V. vulnificus*, *V. alcaligenes* and *V. alginolyticus* (AL-Haj et al., 2010; Manilal et al., 2010; Mariana et al., 2009; Villasin and Pmory, 2000). Another work by Haug et al. (2002b) found

Table 2. Antimicrobial activity in extracts from sea cucumber (*I. badionotus*) against *Vibrio cholerae* (*V. c.*), *Vibrio parahaemolyticus* (*V. p.*), *Vibrio alginolyticus* (*V. a.*), *Vibrio mimicus* (*V. m.*) and *Vibrio vulnificus* (*V. v.*).

Órgan /tissue	Fraction	Antimicrobial activity				
		V. c.	***V. p.***	***V. a.***	***V. m.***	***V. v.***
Body	CE	+	+	+	+	+
	SP	+	+	+	+	+
	MP	++	++	++	++	++
Respirotory organs	CE	-	-	-	-	-
	SP	-	-	-	-	-
	MP	+	++	++	+	++
Gonads	CE	-	-	nt	-	nt
	SP	-	-	Nt	-	Nt
	MP	-	-	Nt	-	Nt

Abbreviations: -, no antimicrobial activity at a protein concentration of 2.6 mg/ml; + antimicrobial activity at a protein concentration of 2.6 mg/ml; ++ antimicrobial activity at a protein concentration of 1 mg/ml; nt, not tested, crude extract (CE), soluble protein (SP), membranal extracts (MP).

Table 3. Minimum inhibitory concentrations (MIC) of *Isostichopus badionotus* aqueous extracts against gram-positive and gram-negative bacterial growth.

Microorganism	MIC MP fraction of body (µg/ml)
S. aureus	37.4
V. cholerae	8.4

antimicrobial activity in muscle extracts of *C. frondosa* against the bacterium *V. glutamicum*. Antimicrobial compounds were also found in ethanol extracts of *H. scabra*, *A. miliaris* and *H. atra*, which inhibited the growth of *S. aureus*, *A. hydrophilla*, *S. typhi* and *E. coli* (Jawahar et. al., 2002). Ridzwan et al. (1995) found that aqueous extracts of *H. atra* and *B. argus* inhibitory effect on the growth of the bacteria *E. coli*, *S. faecalis*, *S. sonnei*, *P. mirabilis* and *S. aureus*. A similar result presented in our study, which noted the presence of antibacterial compounds in aqueous extracts of sea cucumber *I. badionotus*, inhibiting a wide range of pathogenic bacteria. Also found in the respiratory tract of sea cucumbers presence of antibacterial factors, which inhibited the growth of certain microorganisms. In contrast, Haug et al. (2002a) analyzed in acetonitrile extracts of respiratory organs and observed a negative effect on the antibacterial activity. This could be due to their natural habits of feeding sea cucumbers, and microorganisms that feed on living together with the organic content in the seabed (Ridzwan et al., 1995). On the other hand, it is unknown whether these factors are the same antibacterial in all organs tested. But they have isolated a variety of antimicrobial compounds in echinoderm, such as steroidal glycosides, polyhydroxylated sterols, naphthoquinone pigments, lysozyme, antimicrobial peptides and proteins (Haug et al., 2002b). These compounds may be present in aqueous extracts of *I. badionotus*. Therefore, these antibacterial factors could play an important role as a first line of defense to pathogens.

A fundamental concept in testing *in vitro* antimicrobial activity is the determination of minimum inhibitory concentration (MIC). In the MP muscle of *I. badionotus*, the test was conducted and presented an inhibitium of growth in *V. cholerae* in low amounts (8.4 µg ml^{-1}). This was compared by the absence of turbidity in the Trypticase soy broth (TSB). The MIC can be easily affected by the nature of the bacteria, inoculum size, the composition of culture medium, incubation time and incubation conditions, such as temperature, pH and aeration. In this study, the MIC reading was based on measuring the optical density in the culture medium (TSB), which was compared with the control optical density (cells grown in TSB). The MIC of methanol extract of *G. changii* and *S. badionotus* for MRSA is 25 and 7.5 mg/ml, respectively (Al-Haj et al., 2010; Mariana et al., 2009). However in our aqueous extracts (MP), *I. badionotus* presented a growth inhibition of *S. aureus* by adding 37.4 µg/ml protein. In contrast, the isolated *V. cholera* was observed reduction in growth to 8.4 µg/ml protein of MP.

In the treatments with proteinase K and heat of the MP in muscle *I. badionotus*; the antibacterial activity was not affected by such treatment, indicating that the activity detected is given by factors of non-protein nature. A variety of polyhydroxylated steroidal glycosides and sterols with antibacterial activity have been isolated from bodies of Echinoderms (Haug et al., 2002b). Many of these compounds show cytotoxic activity. It is also possible that the antibacterial activity is due to metabolites derived from the diet of the organism. In con-

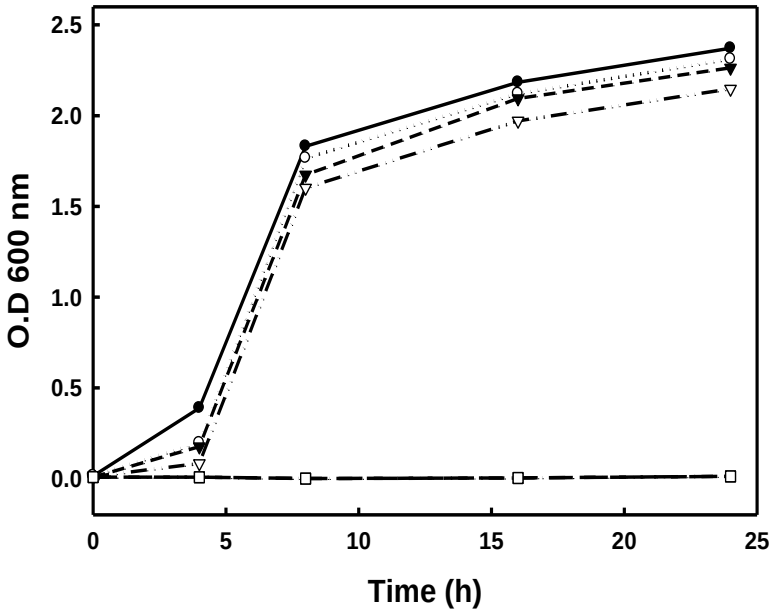

Figure 1. Antibacterial activity of MP body extract from *I. badionotus* against *V. cholerae* grown in TSB. The optical density at 600 nm was measured in a bacterial suspension of 5 x 10^8 cells per tube containing bacteria alone (●), or bacteria plus extract adjusted to a protein concentration of 8.4 mg/ml (○), 16.8 mg/ml (▼), 33.6 mg/ml (▽), and positive control, 0.4 mg/ml ampicillin (□), respectively.

Figure 2. *In vitro* antibacterial activity of MP body extract from *I. badionotus* against *V. cholerae* grown in TSA. 60 µg of protein MP extracts were spotted on discs (2), heat treatment of MP extract (3), proteinase K digest of MP extract (4), as a control (positive) 0.4 mg ml^{-1} ampicillin (5), and extraction buffer (1), respectively.

clusion, this study shows that *I. badionotus* contains factors with antibacterial activity, particularly in muscle and respiratory tract. There were differences in the active fractions of these organs. This study also shows greater antibacterial activity of the PM muscle on *S. aureus* and *V. cholerae* and its resistance to treatment with proteinase K and heat, indicating that the active compound of this fraction is of non-protein source. It is not known if the same antibacterial factor is present in the active fractions, as it has not yet been explored. It is necessary a higher degree of purification of the active compounds to identify their chemical nature and evaluate its potential as new pharmaceutical.

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